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4. Title of the invention

THERAPEUTIC SYSTEMS

5. Name of your agent *(if you have one)*

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ERIC POTTER CLARKSON
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THERAPEUTIC SYSTEMS

The present invention relates to therapeutic systems, particularly therapeutic systems for activating prodrugs and for the use of such systems in killing target cells, particularly tumour cells.

The delivery of a cytotoxic agent to the site of tumour cells is much desired because systemic administration of these agents can result in the killing of normal cells within the body as well as the tumour cells. The resulting toxicity to normal cells limits the dose of the cytotoxic agent and thus reduces the therapeutic potential of these agents. However, in some instances the administered agent has no intrinsic activity but is converted *in vivo* at the appropriate time or place to the active drug. Such analogues are referred to as prodrugs and are used extensively in medicine [Connors and Knox, 1995]. Conversion of the prodrug to the active form can take place by a number of mechanisms depending, for example, on changes of pH, oxygen tension, temperature or salt concentration or by spontaneous decomposition of the drug or internal ring opening or cyclisation.

WO 88/07378 describes a two-component system, and therapeutic uses thereof, wherein a first component comprises an antibody fragment capable of binding with a tumour-associated antigen and an enzyme capable of converting a pro-drug into a cytotoxic drug, and a second component which is a pro-drug which is capable of conversion to a cytotoxic drug. This general system, which is often referred to as "antibody-directed enzyme pro-drug therapy" (ADEPT), is also described in relation to specific enzymes and pro-drugs in EP 0 302 473 and WO 91/11201.

WO 89/10140 describes a modification to the system described in WO

88/07378 wherein a further component is employed in the system. This further component accelerates the clearance of the first component from the blood when the first and second components are administered clinically. The second component is usually an antibody that binds to the antibody-enzyme conjugate and accelerates clearance. An antibody which was directed at the active site on the enzyme had the additional advantage of inactivating the enzyme. However, such an inactivating antibody has the undesirable potential to inactivate enzyme at the tumour sites, but its penetration into tumours was obviated by the addition of galactose residues to the antibody. The galactosylated antibody was rapidly removed from the blood, together with bound antibody-enzyme component, via galactose receptors in the liver. The system has been used safely and effectively in clinical trials. However, galactosylation of such an inactivating antibody which results in its rapid clearance from blood also inhibits its penetration of normal tissue and inactivation of enzyme localised there.

WO 93/13805 describes a system comprising a compound comprising a target cell-specific portion, such as an antibody specific to tumour cell antigens, and an inactivating portion, such as an enzyme, capable of converting a substance which in its native state is able to inhibit the effect of a cytotoxic agent into a substance which has less effect against said cytotoxic agent. The prolonged action of a cytotoxic agent at tumour sites is therefore possible whilst protecting normal tissues from the effects of the cytotoxic agent.

WO 93/13806 describes a further modification of the ADEPT system comprising a three component kit of parts for use in a method of destroying target cells in a host. The first component comprises a target cell-specific portion and an enzymatically active portion capable of converting a pro-drug into a cytotoxic drug; the second component is a

pro-drug convertible by said enzymatically active portion to the cytotoxic drug; and the third component comprises a portion capable of at least partly restraining the component from leaving the vascular compartment of a host when said component is administered to the vascular compartment, and an inactivating portion capable of converting the cytotoxic drug into a less toxic substance.

Our unpublished but co-pending patent application GB 9624993.3 describes a macromolecule prodrug therapy system. Our unpublished but co-pending patent application PCT/GB96/03000 describes the use of enzyme inhibitors in an improvement of ADEPT; and our unpublished but co-pending patent application PCT/GB96/03254 describes the use of internalising antibodies and/or intracellular cofactors in an improvement to ADEPT.

EP 0 415 731 describes a therapeutic system which is often called GDEPT (gene-directed enzyme prodrug therapy).

A major approach in prodrug design is the synthesis of inert analogues which are converted to the active drug by enzyme action. In cancer chemotherapy prodrugs have been used clinically for a variety of purposes ranging from analogues with better formulation properties to prodrugs designed to be selectively activated in the tumour environment. Results from animal experiments and dose intensification studies in humans have indicated that some tumour types, eg ovarian cancer, might be completely eradicated by chemotherapy if the dose of anti-cancer agent to which they respond could be increased by a hundred-fold. Attempts to increase the dose administered using dose intensification, by autologous bone marrow transplantation after high doses of myelotoxic therapy, by rescue experiments eg folinic acid after methotrexate or by isolated limb

perfusions, do allow a greater total dose to be given but not by this order of magnitude. However, there are many examples where this level of dose intensity can theoretically be achieved by using prodrugs which are selectively activated by enzymes present in tumours. Experiments on tumour bearing animals have shown that when a prodrug is activated uniquely in the tumour environment, cures can be obtained for mice bearing large primary tumours and extensive metastases [Connors and Whisson, 1966, Whisson and Connors, 1965]. Given that the prodrug is a good substrate for the enzyme specifically expressed in the tumour and that the difference in toxicity between prodrug and drug is a hundred-fold or more then, once a candidate enzyme has been identified (especially if there is also a high concentration of the enzyme in the extracellular spaces of the tumour), many different classes of anti-cancer agent can often be derivatised to form appropriate prodrugs. This can be demonstrated in approaches used to design prodrugs of cytotoxic alkylating agents. Because this class of anti-cancer agent acts predominantly but probably not exclusively by covalent alkylation of adjacent strands of DNA the first basic requirement for cytotoxicity is that the agent should have an optimal level of chemical reactivity which enables it to reach the tumour site after injection and be reactive enough to alkylate DNA. If the reagent is too reactive it may hydrolyse before reaching the tumour and if too unreactive may be excreted before sufficient DNA alkylation has taken place. Secondly, it must be able to pass through the endothelium and the cell and nuclear membranes to reach its target. Finally, because the predominant reaction that leads to cytotoxicity is a cross-linking reaction, then the alkylating agent must have a minimum of two alkylating arms.

In order to design an appropriate prodrug, once a unique tumour enzyme has been identified, a prodrug is synthesised which is lacking one or more of the features described but is acted upon by the enzyme to produce an

appropriate drug. Thus, many alkylating prodrugs are chemically unreactive and non-toxic but are substrates for enzymes which metabolise them to highly reactive and toxic products. The ability of an alkylating agent to react with biological molecules depends on a minimal level of chemical reactivity and this level of activity can vary greatly depending on chemical structure. Small changes in electron donating or withdrawing properties can greatly alter chemical reactivity. Large numbers of anti-tumour alkylating agents have been tested experimentally and, almost without exception, active derivatives must be at least difunctional, ie have at least two alkylating arms.

Monofunctional agents, although they may be carcinogens, usually are much less toxic and if they can be converted enzymatically to difunctional agents might be effective prodrugs. An example of this is CB 1954 a monofunctional aziridine which was highly effective against the rat Walker tumour which is normally only sensitive to difunctional alkylating agents (reviewed by Knox *et al*, 1993). This tumour has a relatively high concentration of the enzyme DT-diaphorase (NQO1, EC 1.6.99.2) which reduces the 4-nitro group to a hydroxylamine which is then converted (probably by acetyl CoA) to a difunctional agent (Figure 1). However, the human form of DT-diaphorase reduces CB 1954 much more slowly than the rat form and human tumours (even those containing the same levels of DT-diaphorase as rat Walker tumour) are resistant to this agent [Knox *et al*, 1993]. The difference in reduction rate is mostly due to a single amino-acid change, a glutamine to a tyrosine at amino acid position 104 [Chen *et al*, 1997]. Given the provenance of CB 1954 against rat tumours, a number of ways of activating CB 1954 in human tumours have been suggested. The first is antibody directed enzyme prodrug therapy (ADEPT, as mentioned above) in which an antibody is used to localise an *E. coli* nitroreductase at a tumour. This nitroreductase can reduce CB

1954 much more rapidly than rat DT-diaphorase. The system is described in WO 93/08228. Gene directed enzyme prodrug therapy (GDEPT) is a method by which the gene encoding for the nitroreductase from *Escherichia coli* is expressed in tumour cells and thus confers sensitivity to CB 1954. This is described in WO 95/12678.

It has also been reported that CB 1954 cytotoxicity can be dramatically increased in human cells by stimulating their endogenous DT-diaphorase with NRH [Friedlos *et al*, 1992a]. In these experiments, the toxicity of CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide) towards human cells was greatly enhanced by NADH (when foetal calf serum was present in the culture medium) and by nicotinamide riboside (reduced) (NRH), but not by nicotinate riboside (reduced). Co-treatment of human cells with CB 1954 and NADH resulted in the formation of crosslinks in their DNA. The toxicity produced by other DNA crosslinking agents was unaffected by reduced nicotinamide compounds. When caffeine was included in the medium a reduction of the cytotoxicity of CB 1954 occurred. The toxicity experienced by human cell lines after exposure to CB 1954 and NADH was proportional to their levels of the enzyme DT diaphorase. It was concluded that NRH, which has been shown to be a cofactor for rat DT diaphorase [Friedlos *et al*, 1992b], is generated from NADH by enzymes in foetal calf serum [Friedlos and Knox, 1992] and stimulates the activity of human DT diaphorase towards CB 1954. However, it has recently been shown that there is an additional CB1954-reducing activity detectable in human cells in the presence of NRH and that this activity is much greater than that attributable to DT-diaphorase [Quinn, 1996].

The terms "nicotinamide mononucleoside-reduced", "dihydronicotinamide riboside", "nicotinamide riboside (reduced)" and "NRH" are all equivalent and are used interchangeably in the patent specification.

Nicotinamide riboside may be produced enzymatically from its commercially available mononucleotides using methods well known in the art, including those described in Friedlos & Knox (1992) *Biochem. Pharmacol.* **44**, 631-635 which is incorporated herein by reference.

Human NAD(P)H:quinone oxidoreductase2 (NQO2) was identified by its homology to DT-diaphorase (NQO1) [Jaiswal *et al*, 1990]. The last exon in the NQO2 gene is 1603 bp shorter than the last exon of the NQO1 gene and encodes for 58 amino acids as compared to 101 amino acids encoded by the NQO1 gene. This makes the NQO2 protein 43 amino acids shorter than the NQO1 protein. The high degree of conservation between NQO2 and NQO1 gene organization and sequence confirmed that the NQO2 gene encoded for a second member of the NQO gene family in humans but it lacked the quinone-reductase activity characteristic of DT-diaphorase [Jaiswal, 1994]. The NQO2 cDNA-derived protein expressed in monkey kidney COS1 cells efficiently catalyzed nitroreduction of CB 10-200, an analogue of CB 1954 [Jaiswal, 1994]. Northern blot analysis indicated that the NQO2 gene was expressed in human heart, brain, lung, liver, and skeletal muscle but did not express in placenta. In contrast, the NQO1 gene was expressed in all human tissues. Large variations were noticed for expression of the NQO2 and NQO1 genes among various tissues [Jaiswal, 1994].

We have now shown that NQO2 can rapidly reduce CB1954 and consider this enzyme, not DT diaphorase, to be responsible for the potentiating effects of NRH on CB1954 cytotoxicity toward human cells reported by Friedlos *et al* [1992a].

Although all of the aforementioned methods of killing a target cell, such as a tumour cell, in an animal body are useful, it is still desirable to

provide new systems of treatment.

A first aspect of the invention provides a compound comprising a target cell-specific portion and (a) human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug, or (b) a polynucleotide encoding said NQO2 or said variant or fragment or fusion or derivative.

The entity which is recognised by the target cell-specific portion may be any suitable entity which is expressed by tumour cells, virally-infected cells, pathogenic microorganisms, cells introduced as part of gene therapy or normal cells of the body which one wishes to destroy for a particular reason. The entity should preferably be present or accessible to the targeting portion in significantly greater concentrations in or on cells which are to be destroyed than in any normal tissues of the host that cannot be functionally replaced by other therapeutic means. Use of a target expressed by a cancer cell would not be precluded, for example, by its equal or greater expression on an endocrine tissue or organ. In a life-saving situation the organ could be sacrificed provided its function was either not essential to life, for example in the case of the testes, or could be supplied by hormone replacement therapy. Such considerations would apply, for instance, to the thyroid gland, parathyroids, adrenal cortex and ovaries.

The entity which is recognised will often be an antigen. Tumour-associated antigens, when they are expressed on the cell membrane or secreted into tumour extra-cellular fluid, lend themselves to the role of targets for antibodies.

The antigen-specific portion may be an entire antibody (usually, for convenience and specificity, a monoclonal antibody), a part or parts thereof (for example an Fab fragment or $F(ab')_2$) or a synthetic antibody or part thereof. A conjugate comprising only part of an antibody may be advantageous by virtue of optimizing the rate of clearance from the blood and may be less likely to undergo non-specific binding due to the Fc part. Suitable monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H. Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J.G.R. Hurrell (CRC Press, 1982). All references mentioned in this specification are incorporated herein by reference. Bispecific antibodies may be prepared by cell fusion, by reassociation of monovalent fragments or by chemical cross-linking of whole antibodies, with one part of the resulting bispecific antibody being directed to the cell-specific antigen and the other to the enzyme. The bispecific antibody can be administered bound to the enzyme or it can be administered first, followed by the enzyme. It is preferred that the bispecific antibodies are administered first, and after localization to the tumour cells, the enzyme is administered to be captured by the tumour localized antibody. Methods for preparing bispecific antibodies are disclosed in Corvalan *et al* (1987) *Cancer Immunol. Immunother.* **24**, 127-132 and 133-137 and 138-143, and Gillsland *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7719-7723.

The variable heavy (V_H) and variable light (V_L) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody

(Morrison *et al* (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains is known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science* **240**, 1038); single-chain Fv (ScFv) molecules where the V_H and V_L partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-299.

By "ScFv molecules" we mean molecules wherein the V_H and V_L partner domains are linked via a flexible oligopeptide.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent". By "bivalent" we mean that the said antibodies and $F(ab')_2$ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are

monovalent, having only one antigen combining sites. Fragmentation of intact immunoglobulins to produce $F(ab')_2$ fragments is disclosed by Harwood *et al* (1985) *Eur. J. Cancer Clin. Oncol.* **21**, 1515-1522.

IgG class antibodies are preferred.

Alternatively, the entity which is recognised may or may not be antigenic but can be recognised and selectively bound to in some other way. For example, it may be a characteristic cell surface receptor such as the receptor for melanocyte-stimulating hormone (MSH) which is expressed in high numbers in melanoma cells. The cell-specific portion may then be a compound or part thereof which specifically binds to the entity in a non-immune sense, for example as a substrate or analogue thereof for a cell-surface enzyme or as a messenger.

Considerable work has already been carried out on antibodies and fragments thereof to tumour-associated antigens and antibodies or antibody fragments directed at carcinoembryonic antigen (CEA) and antibodies or their fragments directed at human chorionic gonadotrophin (hCG) can be conjugated to carboxypeptidase G2 and the resulting conjugate retains both antigen binding and catalytic function. Following intravenous injection of these conjugates they localise selectively in tumours expressing CEA or hCG respectively. Other antibodies are known to localise in tumours expressing the corresponding antigen. Such tumours may be primary and metastatic colorectal cancer (CEA) and choriocarcinoma (hCG) in human patients or other forms of cancer. Although such antibody-enzyme conjugates may also localise in some normal tissues expressing the respective antigens, antigen expression is more diffuse in normal tissues. Such antibody-enzyme conjugates may be bound to cell membranes via their respective antigens or trapped by antigen secreted into the interstitial

space between cells.

Examples of tumour-associated, immune cell-associated and infection reagent-related antigens are given in Table 1.

5

TABLE 1: Cell surface antigens for targeting

a) Tumour Associated Antigens

Antigen	Antibody	Existing uses
Carcino-embryonic Antigen	C46 (Amersham) 85A12 (Unipath)	Imaging and therapy of colon/rectum tumours.
Placental Alkaline Phosphatase	H17E2 (ICRF, Travers & Bodmer)	Imaging and therapy of testicular and ovarian cancers.
Pan Carcinoma	NR-LU-10 (NeoRx Corporation)	Imaging and therapy of various carcinomas including small cell lung cancer.
Polymorphic Epithelial Mucin (Human milk fat globule)	HMFG1 (Taylor-Papadimitriou, ICRF)	Imaging and therapy of ovarian cancer and pleural effusions.
β-human Chorionic Gonadotropin	W14	Targeting of carboxypeptidase to human xenograft choriocarcinoma in nude mice (Searle *et al* (1981) *Br. J. Cancer* **44**, 137-144).
A carbohydrate on Human Carcinomas	L6 (IgG2a)[1]	Targeting of alkaline phosphatase (Senter *et al* (1988) *PNAS USA* **85**, 4842-4846.
CD20 Antigen on B Lymphoma (normal and neoplastic)	1F5 (IgG2a)[2]	Targeting of alkaline phosphatase (Senter *et al* (1988) *PNAS USA* **85**, 4842-4846.

[1]Hellström *et al* (1986) *Cancer Res.* **46**, 3917-3923

[2]Clarke *et al* (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1766-1770

Other antigens include alphafoetoprotein, Ca-125 and prostate specific antigen.

b) Immune Cell Antigens

Antigen	Antibody	Existing uses
Pan T Lymphocyte Surface Antigen (CD3)	OKT-3 (Ortho)	As anti-rejection therapy for kidney transplants.
B-lymphocyte Surface Antigen (CD22)	RFB4 (Janossy, Royal Free Hospital)	Immunotoxin therapy of B cell lymphoma.
Pan T lymphocyte Surface Antigen (CD5)	H65 (Bodmer and Knowles, ICRF; licensed to Xoma Corp., USA)	Immunotoxin treatment of acute graft versus host disease, rheumatoid arthritis.

c) Infectious Agent-Related Antigens

Antigen	Antibody	Existing uses
Mumps virus-related	Anti-mumps polyclonal antibody	Antibody conjugated to diphtheria toxin for treatment of mumps.
Hepatitis B Surface Antigen	Anti HBs Ag	Immunotoxin against hepatoma.

Other tumour selective targets and suitable binding moieties are shown in Table 2.

Table 2: Binding moieties for tumour-selective targets and tumour-associated antigens

Target	Binding moiety	Disease
Truncated EGFR	anti-EGFR mAb	Gliomas
Idiotypes	anti-id mAbs	B-cell lymphomas
EGFR (*c-erb*B1)	EGF, TGFα anti-EGFR mAb	Breast cancer
*c-erb*B2	mAbs	Breast cancer
IL-2 receptor	IL-2 anti-Tac mAb	Lymphomas and leukaemias
IL-4 receptor	IL-4	Lymphomas and leukaemias
IL-6 receptor	IL-6	Lymphomas and leukaemias
MSH (melanocyte-stimulating hormone) receptor	α-MSH	Melanomas
Transferrin receptor (TR)	Transferrin anti-TR mAb	Gliomas
gp95/gp97	mAbs	Melanomas
p-glycoprotein cells	mAbs	drug-resistant
cluster-1 antigen (N-CAM)	mAbs	Small cell lung carcinomas
cluster-w4	mAbs	Small cell lung carcinomas
cluster-5A	mAbs	Small cell lung carcinomas
cluster-6 (LeY)	mAbs	Small cell lung carcinomas

PLAP (placental alkaline phosphatase)	mAbs	Some seminomas Some ovarian; some non small cell lung cancer
CA-125	mAbs	Lung, ovarian carcinoma
ESA (epithelial specific antigen)	mAbs	
CD 19, 22, 37	mAbs	B-cell lymphomas
250 kDa	mAbs	Melanoma
proteoglycan p55	mAbs	Breast cancer
TCR-IgH fusion	mAbs	Childhood T-cell leukaemia
Blood gp A antigen (in B or O individuals)	mAbs	Gastric and colon tumours
Mucin protein core	mAbs	Breast cancer

It is preferred if the target cell-specific portion comprises an antibody or fragment or derivative thereof.

The target cell-specific portion may, however, be any compound which leads to the accumulation of the NQO2 or a said variant or fragment or fusion or derivative thereof at the site of the target cell (such as a tumour). For example, non-specific uptake of a macromolecule by a tumour is enough to deliver an appropriate level of the enzyme in an ADEPT-type of system and an adequate ratio of tumour-associated cytotoxic drug to non-tumour-associated drug can be achieved if the enzyme-macromolecule conjugate is cleared or inhibited when away from the tumour. This approach is applicable to any of the ADEPT systems described above but should perhaps be called MDEPT (macromolecule directed enzyme prodrug therapy).

The term "tumour" is to be understood as referring to all forms of neoplastic cell growth, including tumours of the lung, liver, blood cells, skin, pancreas, stomach, colon, prostate, uterus, breast, lymph glands and bladder. Solid tumours are especially suitable.

The potential advantages in using non-antibody macromolecules for this purpose are substantial. Firstly, a non-specific macromolecule may be selected that is non-immunogenic. Secondly, a macromolecule may be much less costly to produce than humanised antitumour antibody. Thirdly, it has been shown that some polymers reduce or eliminate the immunogenicity of proteins, including enzymes, attached to them (Abuchowski *et al*, 1977, Wileman *et al*, 1986, Mikolajczyk *et al*, 1996). Fourthly, whereas an antibody binds to only a limited range of cancers (antibodies only exist for about 60% of all malignancies), the macromolecule uptake by tumours appears to be a characteristic common to all solid cancers so far examined.

Thus, many tumours such as sarcomas for which no selective antibodies have yet been reported may be targeted using this principle.

The relatively low differential between tumour and non-tumour tissues with non-specific macromolecules is exploitable only if the level of normal tissue enzyme is inhibited, for example by using a galactosylated anti-enzyme antibody. To get the required amount of enzyme to tumour sites when the enzyme is conjugated to a non-specific macromolecule may require a greater amount of such a conjugate to be administered than would be the case with a specific antibody-enzyme conjugate, but the lower cost of the former should offset its lower efficiency.

Preferably, the macromolecule used in the invention is hydrophilic and is

characterised by being soluble in body fluids and in conventional fluids for parenteral administration. Suitably, the macromolecule is biodegradable so that systemic accumulation during repeated administration is avoided. Clearly, however, it must not be degraded so fast as to fail to accumulate at the tumour site. Preferably, when conjugated to the selected enzyme, the molecular weight and size of the conjugate should exceed that of the renal threshold for urinary excretion (MW 60 000), as this helps the blood concentration to be sufficient to provide an effective blood:tumour concentration gradient. A molecular weight of up to at least 800 000 is generally suitable, for example up to 160 000. The macromolecule is preferably one which is not readily captured by the reticuloendothelial system. To make it catalytic, the macromolecule may be conjugated to one or more enzyme molecules by simple chemical methods, using bi-functional agents which do not degrade the attached enzyme. Preferably, the starting macromolecule confers reduced immunogenicity on an immunogenic enzyme to which it is conjugated.

Macromolecules that are available as sub-units and are not biodegradable may be linked by biodegradable linking units so that the non-biodegradable components are filtered through the kidneys and excreted in the urine.

Whereas some macromolecules are not known to be internalised by cells others, such as N-(2-hydroxypropyl)methylacrylamide, are internalised through more than one mechanism (Duncan *et al*, 1996).

Preferably, the macromolecule is polyethylene glycol (PEG). Derivatisation of proteins with polyethylene glycol has been demonstrated numerous times to increase their blood circulation lifetimes as well as decrease their antigenicity and immunogenicity.

MDEPT is described in more detail in our patent application GB 9624993.3 incorporated herein by reference.

Thus, a preferred embodiment for delivery of the enzyme to tumour sites is to take advantage of the leakiness of tumour capillaries and the poor lymphatic drainage of tumours. Thus, it has been shown that an enzyme conjugated to form a macromolecule, for instance by conjugation to polyethylene glycols or dextrans is selectively taken up by tumours.

The cDNA encoding human NAD(P)H:quinone reductase 2 (NQO2) is given in Jaiswal *et al* (1990) *Biochemistry* **29**, 1899-1906 and the gene structure of the NQO2 gene is given in Jaiswal (1994) *J. Biol. Chem.* **269**, 14502-14508, both of which are incorporated herein by reference and the nucleotide sequence and encoded amino acid sequence is given in Figure 6. The skilled person can readily obtain and manipulate DNA encoding NQO2 based on the teachings contained herein using genetic engineering and recombinant DNA techniques which are well known, some of which are described in Sambrook *et al* (1989), "Molecular cloning, a laboratory manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

A variant or fragment or fusion or derivative of NQO2 may be used in place of NQO2 with the given amino acid sequence provided that it has substantially the same activity as NQO2 towards a given prodrug. The enzyme NQO2 catalyses the conversion of, for example, the prodrug CB 1954 and prodrug analogues thereof. Thus, preferably the variant or fragment or fusion or derivative of NQO2 has substantially the same activity towards CB 1954 as does NQO2 itself. Conveniently, the said variant or fragment or fusion or derivative has at least 0.1 x the k_{cat}/K_m of NQO2, more preferably at least 0.5 x and still more preferably at least 0.9

x the k_{cat}/K_m of NQO2.

Preferably, the variant or fragment or fusion or derivative of NQO2 also has substantially the same cofactor specificity. Preferably the variant or fragment or fusion or derivative of NQO2 can use nicotinamide riboside (reduced) (NRH) as a cofactor. Preferably, the variant or fragment or fusion or derivative of NQO2 binds the cofactor at least 0.1 x as well as NQO2 itself, more preferably at least 0.5 x as well and still more preferably at least 0.9 x as well.

By a "variant" we include polypeptides in which one or more amino acids have been replaced or deleted. Typically, the variant has amino acid conservative replacements in which, for example, the following groups of amino acid may be interchanged: Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr, Lys, Arg; and Phe, Tyr. Such variants may be made using the methods of protein engineering and site-directed mutagenesis. The term "variants" also includes polypeptides with insertions and deletions.

By a "fragment" we mean a portion of NQO2 provided that it retains substantially the same activity as NQO2 towards a given prodrug.

By a "fusion" we mean a fusion of NQO2 or a variant or fragment thereof to any other polypeptide, for example, in some circumstances it may be desirable to fuse NQO2 or a variant or fragment thereof to another polypeptide which can facilitate purification. This may be, for example, glutathione-S-transferase, or the well known Myc tag sequence or His_n where $n>4$. In each case the additional polypeptide allows the fusion to be purified by affinity chromatography.

When the two portions of the compound of the first aspect of the invention

are polypeptides they may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al* (1979) *Anal. Biochem.* **100**, 100-108. For example, an antibody portion may be enriched with thiol groups and the enzyme portion reacted with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with m-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

It may not be necessary for the whole NQO2 to be present in the compound of the first aspect of the invention but, of course, the catalytic portion must be present.

Alternatively, the compound may be produced as a fusion compound by recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two portions of the compound of the invention either adjacent to one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the compound. Conceivably, the two portions of the compound may overlap wholly or partly. The antibody (or other polypeptide portion which targets a cell) component of the fusion must be represented by at least one binding site. Examples of the construction of antibody (or antibody fragment)-enzyme fusions are disclosed by Neuberger *et al* (1984) *Nature* **312**, 604.

The DNA is then expressed in a suitable host to produce a polypeptide comprising the compound of this aspect of the invention. Thus, the DNA encoding the polypeptide constituting the compound of this aspect of the

invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA encoding the polypeptide constituting the compound of this aspect of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the

expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a procaryotic replicon, such as the ColE1 *ori*, for propagation in a procaryote, even if the vector is to be used for expression in other, non-procaryotic, cell types. The vectors can also include an appropriate promoter such as a procaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical procaryotic vector plasmids are pUC18, pUC19, pBR322 and

pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *his3, trp1, leu2* and *ura3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

A variety of methods have been developed to operatively link DNA to vectors via complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described

earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3′-single-stranded termini with their 3′-5′-exonucleolytic activities, and fill in recessed 3′-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of this aspect of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491.

In this method the DNA to be enzymatically amplified is flanked by two specific oligonucleotide primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

Exemplary genera of yeast contemplated to be useful in the practice of the

present invention are *Pichia, Saccharomyces, Kluyveromyces, Candida, Torulopsis, Hansenula, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschunikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis*, and the like. Preferred genera are those selected from the group consisting of *Pichia, Saccharomyces, Kluyveromyces, Yarrowia* and *Hansenula*. Examples of *Saccharomyces* are *Saccharomyces cerevisiae*, *Saccharomyces italicus* and *Saccharomyces rouxii*. Examples of *Kluyveromyces* are *Kluyveromyces fragilis* and *Kluyveromyces lactis*. Examples of *Hansenula* are *Hansenula polymorpha, Hansenula anomala* and *Hansenula capsulata*. *Yarrowia lipolytica* is an example of a suitable *Yarrowia* species.

Methods for the transformation of *S. cerevisiae* are taught generally in EP 251 744, EP 258 067 and WO 90/01063, all of which are incorporated herein by reference.

Suitable promoters for *S. cerevisiae* include those associated with the *PGK1* gene, *GAL1* or *GAL10* genes, *CYC1*, *PHO5*, *TRP1*, *ADH1*, *ADH2*, the genes for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, triose phosphate isomerase, phosphoglucose isomerase, glucokinase, α-mating factor pheromone, a-mating factor pheromone, the *PRB1* promoter, the *GUT2* promoter, and hybrid promoters involving hybrids of parts of 5′ regulatory regions with parts of 5′ regulatory regions of other promoters or with upstream activation sites (eg the promoter of EP-A-258 067).

The transcription termination signal is preferably the 3′ flanking sequence of a eukaryotic gene which contains proper signals for transcription termination and polyadenylation. Suitable 3′ flanking sequences may, for example, be those of the gene naturally linked to the expression control

sequence used, ie may correspond to the promoter. Alternatively, they may be different in which case the termination signal of the *S. cerevisiae AHD1* gene is preferred.

By "polynucleotide encoding said NQO2 or said variant or fragment or fusion or derivative" we include any such polynucleotide. The polynucleotide may be RNA or DNA; preferably it is DNA.

It will be appreciated that when the compound of the first aspect of the invention comprises a polynucleotide encoding human NQO2 or a polynucleotide encoding a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug the target cell-specific portion of the compound is one which is adapted to deliver the polynucleotide (genetic construct) to the target cell.

Preferably, the genetic construct is adapted for delivery to a cell, preferably a human cell. More preferably, the genetic construct is adapted for delivery to a cell in an animal body, more preferably a mammalian body; most preferably it is adapted for delivery to a cell in a human body.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the tumour cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the tumour cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviruses provide a potential means of selectively infecting cancer cells because they can only integrate into the genome of dividing cells; most normal cells surrounding cancers are in a quiescent, non-receptive stage of cell growth or, at least, are dividing much less

rapidly than the tumour cells. Retroviral DNA constructs which contain a suitable promoter segment and a polynucleotide encoding NQO2 or a variant or fragment or fusion or derivative as defined may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a *neo*^{R} gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the tumour cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. For tumours exceeding 10 mm in diameter it is appropriate to inject between 0.1 ml and 1 ml of retroviral supernatant; preferably 0.5 ml.

Alternatively, as described in Culver *et al* (1992) *Science* **256**, 1550-1552, cells which produce retroviruses are injected into the tumour. The retrovirus-producing cells so introduced are engineered to actively produce retroviral vector particles so that continuous productions of the vector occurred within the tumour mass *in situ*. Thus, proliferating tumour cells can be successfully transduced *in vivo* if mixed with retroviral vector-producing cells.

Targeted retroviruses are also available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into preexisting viral *env* genes (see Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for

gene therapy).

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes (preferably tumour-cell-targeted) liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653).

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cancer cell types which over-express a cell surface protein for which antibodies are available (see Table for examples). For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)-butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected intraperitoneally or directly into the tumour.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA via electrostatic interactions with the phosphate backbone. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the tumour cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse a tumour with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible tumours.

It will be appreciated that "naked DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* 6, 1129-1144.

Thus, it will be appreciated that a further aspect of the invention provides a composition comprising genetic construct as defined in the invention and

means for introducing said genetic construct into a cell, preferably the cell of an animal body.

Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

It will be appreciated that in the first aspect of the invention the polynucleotide need not be one which has a target cell-specific promoter to drive the expression of NQO2 or said variant or fragment or fusion or derivative thereof since the compound comprises a target cell-specific portion as described above for targeting the polynucleotide to the target cell. However, it may be advantageous if the polynucleotide comprises a target cell-specific promoter operably linked to a polynucleotide encoding human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug.

It will be further appreciated that target cell-specific expression of NQO2 or the said variants, fragments, fusions or derivatives may be achieved using a polynucleotide or genetic construct comprising a target cell-

specific promoter whether or not the polynucleotide or genetic construct is comprised in a compound of the first aspect of the invention.

Thus, a second aspect of the invention provides a recombinant polynucleotide comprising a target cell-specific promoter operably linked to a polynucleotide encoding human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug.

Preferably the target cell-specific promoter is a tumour cell-specific promoter.

Useful genetic elements which are target cell-specific promoters are given below but new ones are being discovered all of the time which will be useful in this embodiment of the invention.

The tyrosinase and TRP-1 genes both encode proteins which play key roles in the synthesis of the pigment melanin, a specific product of melanocytic cells. The 5′ ends of the tyrosinase and tyrosinase-related protein (TRP-1) genes confer tissue specificity of expression on genes cloned downstream of these promoter elements.

The 5′ sequences of these genes are described in Bradl, M. *et al* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 164-168 and Jackson, I.J. *et al* (1991) *Nucleic Acids Res.* **19**, 3799-3804.

Prostate-specific antigen (PSA) is one of the major protein constituents of the human prostate secretion. It has become a useful marker for the detection and monitoring of prostate cancer. The gene encoding PSA and its promoter region which directs the prostate-specific expression of PSA

have been described (Lundwall (1989) *Biochem. Biophys. Res. Comm.* **161**, 1151-1159; Riegman *et al* (1989) *Biochem. Biophys. Res. Comm.* **159**, 95-102; Brawer (1991) *Acta Oncol.* **30**, 161-168).

Carcinoembryonic antigen (CEA) is a widely used tumour marker, especially in the surveillance of colonic cancer patients. Although CEA is also present in some normal tissues, it is apparently expressed at higher levels in tumorous tissues than in corresponding normal tissues. The complete gene encoding CEA has been cloned and its promoter region analysed. A CEA gene promoter construct, containing approximately 400 nucleotides upstream from the translational start, showed nine times higher activity in the adenocarcinoma cell line SW303, compared with the HeLa cell line. This indicates that *cis*-acting sequences which convey cell type specific expression are contained within this region (Schrewe *et al* (1990) *Mol. Cell. Biol.* **10**, 2738-2748).

The mucin gene, MUC1, contains 5′ flanking sequences which are able to direct expression selectively in breast and pancreatic cell lines, but not in non-epithelial cell lines as taught in WO 91/09867.

The alpha-fetoprotein (AFP) enhancer may be useful to drive pancreatic tumour-selective expression (Su *et al* (1996) *Hum. Gene Ther.* 7, 463-470).

The genetic constructs of the invention can be prepared using methods well known in the art.

A third aspect of the invention provides a therapeutic system comprising a compound of the first aspect of the invention or a polynucleotide of the second aspect of the invention and a prodrug which is converted to a

substantially cytotoxic drug by the action of NQO2.

Suitable prodrugs are described in Mauger *et al* (1994) *J. Med. Chem.* 37, 3452-3458, incorporated herein by reference, and include 4-nitrobenzyloxycarbonyl derivatives of actinomycin D, mitomycin C, doxorubicin, 4-[bis(2-chloroethyl)amino]aniline and 4-[bis(2-chloroethyl)amino]phenol. Upon enzymatic reduction the active drug is generated through self immolation of the 4-(hydroxyamino)benzyloxy carbonyl group.

Preferably the prodrug is CB 1954 or an analogue thereof; most preferably the prodrug is CB 1954.

Analogues of CB1954 are suitably defined as molecules which retain the essential structural features of CB1954 ie a benzene ring containing an aziridine ring, two NO_2 groups and another substituent R but which differ in either the relative orientation of the substituents and/or in the nature of R. A number of analogues have been disclosed in Khan A.H. and Ross W.C.J. (1969) *Chem. Biol. Interact.* **1**, 27-47 and in Khan A.H. and Ross W.C.J. (1971) *Chem. Biol. Interact.* **4**, 11-22, both of which are incorporated herein by reference and in particular the details of the analogues of CB1954 are included in this description.

Preferably the therapeutic system further comprises NRH or an analogue thereof which is able to pass reducing equivalents to NQO2. Suitable analogues include the reduced form of 1-methylnicotinamide and others which are also described in Friedlos *et al* (1992b) and Knox *et al* (1995), both of which are incorporated herein by reference.

A fourth aspect of the invention provides a method of treating a patient

with a target cell to be destroyed the method comprising (a) administering to the patient a compound of the first aspect of the invention or a recombinant polynucleotide of the second aspect of the invention; (b) allowing the NQO2 or a variant or fragment or fusion or derivative thereof to localize at, or be expressed in, the target cell; and (c) administering a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2. It is particularly preferred if NRH or another suitable cofactor of NQO2 is administered to the patient. Another suitable cofactor of NQO2 includes analogues of NRH which are able to pass reducing equivalents to NQO2 and includes molecules which are able to bind NQO2 and pass reducing equivalents to NQO2 substantially as NRH. The administration of the cofactor may be before or after or at the same time as administration of the prodrug.

It is particularly preferred if NRH or an analogue thereof is administered before the prodrug.

Thus, the method is useful in destroying a target cell in a host (eg patient). Preferably, the patient to be treated has a tumour.

The prodrug may be any suitable prodrug as described above.

Preferably the prodrug is CB 1954 or an analogue thereof.

Preferably, when the compound is one comprising a target cell-specific portion and human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug, the compound is administered and, once there is an optimum balance between the target cell to normal cell ratio of the compound and the absolute level of compound associated

with the target, the prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 is administered. The interval between the administration of the compound and the prodrug will depend on the target cell localisation characteristics of the particular compound, but typically it will be between 6 and 48 hours.

Suitably, prodrug administration commences as soon as the plasma activity of enzyme and, by inference, the activity in normal tissues, is insufficient to catalyse enough prodrug to cause toxicity.

Thus, in a preferred embodiment, NQO2 is conjugated to a monoclonal antibody directed at a tumour-associated antigen so as to localise at tumour sites and CB1954 given when the enzyme has cleared from blood and normal tissues. As discussed above, it is particularly preferred if NRH or another suitable cofactor of NQO2 is administered to the patient. Preferably, NRH or an analogue thereof is administered before the prodrug.

Preferably, when the compound is one comprising a target cell-specific portion and a polynucleotide encoding human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug, the compound is administered and, once the NQO2 or a said variant or derivative or fusion or fragment thereof is expressed in the target cell to a useful extent, the prodrug is administered. As discussed above it is particularly preferred if NRH or another suitable cofactor of NQO2 is administered to the patient. Preferably, NRH or an analogue thereof is administered before the prodrug.

In this embodiment, the interval between the administration of the

compound and the prodrug will depend on the target cell localisation characteristics of the particular compound but also on the expression characteristics of the polynucleotide in the particular target cell.

Preferably, then a recombinant polynucleotide of the second aspect of the invention is administered in the method of treatment of the invention, the recombinant polynucleotide is expressed in the target cells to produce NQO2 or a said variant or derivative or fragment or fusion thereof and when the expression is at a useful level, the prodrug is administered. As discussed above, it is particularly preferred if NRH or another suitable cofactor of NQO2 is administered to the patient. Preferably, NRH or an analogue thereof is administered before the prodrug.

Thus, the cytotoxic drug is released in relatively high concentration at the target or tumour site but not at non-target or non-tumour sites.

It will be appreciated that it is not necessary for the compound of the invention to locate to, or the polynucleotide of the invention to be expressed in, all target cells but that the compound should locate to, or the polynucleotide be expressed in, sufficient target cells to have a desirable effect upon administration of the prodrug.

At least with the ADEPT-type embodiment of the invention it may be advantageous to make use of a modification of the system which allows for improved target cell selectivity (especially tumour cell selectivity) by clearing antibody-enzyme conjugates from the blood.

The principle of this improvement is described in detail in WO 89/10140, incorporated herein by reference. Thus, clearance of residual enzyme activity from blood and normal tissues can be accelerated thereby

maximising the tumour to normal tissue ratio of enzyme. Accelerated clearance has been achieved, for example, by means of a monoclonal antibody directed at any part of the antibody-enzyme conjugate but is especially effective when the anti-enzyme antibody inactivates the enzyme. To avoid the anti-enzyme antibody inactivating enzyme at tumour sites it can be galactosylated which results in rapid removal of the anti-enzyme-enzyme-antibody complex from the blood by galactose receptors in hepatocytes. This has been described in WO 89/10140 and in Sharma *et al*, 1990.

At least with the ADEPT- and MDEPT- type embodiments of the present invention it may be advantageous to make use of a modification of the system which allows for improved target cell selectivity (especially tumour cell selectivity) by using inhibitors of NQO2. For example, flavones are inhibitors of NQO2. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is the most potent inhibitor tested so far. It is a competitive inhibitor with respect to NRH (K_i= 27nm), and so may be particularly useful. The principle of this improvement is described in detail in our co-pending patent application PCT/GB96/03000, incorporated herein by reference. Thus, an alternative to the use of a second monoclonal antibody for clearance of enzyme from blood and normal tissues is to employ a small molecule which complements the active site of the enzyme but is not a substrate and is sterically bound in the site. Such a molecule has to be administered at a dose level to inactivate enzyme in blood and normal tissues but at a dose level insufficient to inactivate the higher concentration of enzyme in tumour tissues.

At least in the ADEPT-type embodiment of the method of the invention it may be advantageous if the compound is taken up by the target cell such

that the enzyme is present within the target cell. The principles of this improvement are described in our co-pending patent application PCT/GB96/03254, incorporated herein by reference.

The methods of treatment of the fourth aspect of the invention allow for the NQO2 or said derivative or fragment or variant or fusion to be present either within the target cell or outside the target cell. For example, in the embodiments wherein a polypeptide version of NQO2 are administered to the patient the polypeptide may locate either within the target cell (for example, by using the ADEPT system with internalising antibodies) or it may locate outside the target cell (for example, by using the ADEPT system with antibodies which remain substantially outside the target cell).

Similarly, in the embodiments wherein a polynucleotide encoding NQO2 are administered to the patient the polynucleotide may express NQO2 which is retained within the target cell or it may express NQO2 outside of, but associated with, the target cell. External expression of the enzyme may be achieved by linking it to a signal sequence which directs the enzyme to the surface of a mammalian cell. This will normally be a mammalian signal sequence or a derivative thereof which retains the ability to direct the enzyme to the cell surface. Suitable signal sequences include those found in transmembrane receptor tyrosine kinases such as c-erbB2 signal sequence, the sequence of which is published in Coussens *et al* (1985) Science **230**, 1132-1139, incorporated herein by reference.

In those embodiments of the method where NQO2 is located outside the target cell, but nevertheless associated with the target cell, it will be appreciated that cosubstrate need not be permeable to the cell membrane and this is a preferred property of the cosubstrate in this embodiment since there will be no reduction of the prodrug by endogenous, intracellular

NQO2. In this embodiment it is preferred if the prodrug is substantially unable to permeate the cell membrane although it may do so. However, it is believed that the cytotoxic drug should be able to penetrate the cell because it is generally believed that its cytotoxic effect is due to its reactivity within the cell.

It is preferred if the system of the third aspect of the invention further comprises a cosubstrate for NQO2 which is substantially permeable to the target cell membrane.

It is also preferred if the method of the fourth aspect of the invention further comprises administering to the patient an effective amount of a cosubstrate for NQO2 which can substantially permeate to the target cell membrane.

It is preferred if the cosubstrate is NRH or an analogue thereof especially one which can substantially permeate a cell membrane. Suitable analogues include the reduced form of 1-methylnicotinamide and others which are also described in Friedlos *et al* (1992b) and Knox *et al* (1995), both of which are incorporated herein by reference.

Thus, it will be seen that the compound of the first aspect of the invention and the recombinant polynucleotide of the second aspect of the invention are useful in medicine and that they are therefore packaged and presented for use in medicine.

The invention also provides the use of a compound of the first aspect of the invention, or a polynucleotide of the second aspect of the invention, in the manufacture of a medicament for treating a target cell to be destroyed. Preferably the patient has been, is being or will be

administered a prodrug which is converted to a substantially cytotoxic drug by action of NQO2.

The invention also provides the use of a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 in the manufacture of a medicament for treating a patient with a target cell to be destroyed wherein the patient has been, is being or will be administered a compound according to the first aspect of the invention, or a polynucleotide according to the second aspect of the invention.

The invention also provides the use of NRH or an analogue thereof which can pass reducing equivalents to NQO2 in the manufacture of a medicament for treating a patient with a target cell to be destroyed wherein the patient has been, is being or will be a compound according to the first aspect of the invention, or a polynucleotide according to the second aspect of the invention and a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2.

A fifth aspect of the invention provides a method of treating a human patient with a target cell to be destroyed wherein the target cell expresses NQO2 the method comprising administering to the patient a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 and nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

Preferably the target cell expresses NQO2 naturally (for example by virtue of the disease state) but it may be a target cell which has been induced to produce NQO2 or which expresses NQO2 by virtue of induction or manipulation of the cell.

The NRH analogues are those as described above in relation to the previous aspects of the invention.

The prodrugs are those as described above in relation to the previous aspects of the invention. It is particularly preferred if the prodrug is CB1954 or an analogue thereof.

It is particularly preferred if NRH or an analogue thereof which is substantially able to permeate the target cell membrane is used in the method of the fifth aspect of the invention.

Preferably the target cell is a tumour cell. It is particularly preferred to use the method of treatment for tumours which show an elevated level of NQO2 compared to non-tumour tissue.

Co-administration of CB 1954 with the co-factor NRH provides a basis for activation of CB 1954 at intracellular sites where NQO2 is expressed. Present indications are that the enzyme is highly expressed in colorectal cancers. It may also be expressed in some normal tissues and, if so, activation in normal tissues may be dose limiting.

Thus, it is particularly preferred to treat colorectal cancers with the method of the invention.

According to the method of the invention a prodrug and a co-substrate is administered to a tumour-bearing mammalian host. The prodrug, that is much less cytotoxic to tumour cells than the active drug, is converted to its active form by the enzyme human NAD(P)H:quinone oxidoreductase 2 (NQO2) only in the presence of the co-substrate. Prodrugs that are useful in the method of this invention include, but are not limited to, CB

1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). Co-substrates that are useful in the method of this invention include, but are not limited to, NRH (nicotinamide mononucleoside-reduced (dihydronicotinamide riboside)) (Figure 2). It is appreciated that both the prodrug and co-substrate should be substantially capable of permeating the cell membrane. NADH and NMNH are substantially impermeable to cell membranes. However, it will be appreciated that "by administering NRH or an analogue thereof" in relation to this and previous aspects of the invention we include administering a compound which is converted within the body of the patient to NRH or an analogue thereof. It will be appreciated that a further embodiment includes the possibility of administering to the patient a precursor of NRH or an analogue thereof and means for converting the precursor to NRH or an analogue thereof.

According to a preferred embodiment of this invention endogenous NQO2 is used to activate CB 1954 in the presence of NRH (Figure 3). Using *in vitro* enzyme assays it is demonstrated that CB 1954 is reduced to its 4-hydroxylamine derivative (5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide). This reduction is much greater than that by either human or rat DT-diaphorase and is not readily catalysed by either of the biogenic co-substrates, NADH or NADPH.

The method of the fifth aspect of the invention is particularly suited for the treatment of a patient with target cells to be destroyed wherein the target cells express NQO2. Thus, in a particularly preferred embodiment it is determined whether the target cells express NQO2 prior to administration of the prodrug or NRH or an analogue. This determination can be achieved, for example, by measuring NQO2 levels in a sample comprising the target cell. This may be achieved enzymatically or by using probes selective for the NQO2 polypeptide or mRNA.

Conveniently, this can be achieved using the techniques commonly referred to as western and northern blotting, respectively. In the case of the polypeptide the probe may be a mono- or polyclonal antibody raised against the NQO2 protein or a fragment thereof. Such antibodies could also be used to identify NQO2 in tissue sections by using immunocytochemistry and related techniques. Probes against mRNA will be oligonucleotides or DNA fragments complementary to partial sequences of the NQO2 mRNA sequence. Although these methods are preferred, other methods may be used to detect and quantify NQO2 polypeptide or mRNA levels in a target cell or tissue.

The invention therefore also includes a therapeutic system comprising a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 and nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2. It is preferred if the system further comprises means for determining whether the target cell expresses NQO2.

The invention also includes nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2 for use in medicine, use of nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2 in the manufacture of a medicament for treating a human patient with a target cell to be destroyed, and use of a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 in the manufacture of a medicament for treating a human patient with a target cell to be destroyed wherein the patient has been, is being or will be administered NRH or an analogue thereof which can pass reducing equivalents to NQO2.

The invention will now be described in more detail by reference to the

following Examples and Figures wherein

Figure 1 shows the bioactivation of CB 1954. The initial step is the reduction of CB 1954 by the enzyme DT diaphorase to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine derivative can react with thioesters to produce DNA reactive species. It is postulated that this it the N-acetoxy derivative. The major product of this reaction is however 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide that does not react readily with DNA. Formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is in competition with the production of DNA binding products.

Figure 2 shows the structure of NRH.

Figure 3 is a schematic representation of the bioactivation of CB 1954 by NQO2.

Figure 4 shows the reduction of CB 1954 by NQO2 in the presence of various cosubstrates.

Figure 5 shows the formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (4-NHOH) from the reduction of CB 1954 by NQO2.

Figure 6 shows the nucleotide sequence of a cDNA encoding human NQO2 and its deduced amino acid sequence.

Example 1

Experimental details

Recombinant human NQO2 was prepared in *E. coli*. A NcoI and a HindIII restriction site were added to the 5′- and 3′-ends of the full-length NQO2 cDNA, respectively, using a PCR method with primers and nucleotide sequences derived from the 5′ and 3′-ends of the cDNA. The PCR product was resolved over a 1% agarose gel and then extracted using the QIAquick Gel Extraction Kit (Qiagen Inc). The gel-purified PCR product was cloned into PCRII vector from the TA cloning kit (Invitrogen Co) and the correct sequence of the PCR product was checked by dideoxy sequencing. The resulting construct was religated into the *E. coli* expression vector, pKK233-2 (Pharmacia) through the engineered NcoI and HindIII restriction sites. The expression plasmid was designated pKK-hNQO2. The pKK-hNQO2 *E. coli* cells were cultured, sonicated and centrifuged as previously described for the purification of recombinant DT diaphorase [Chen *et al*, 1992]. The supernatant from a 90 min-centrifugation at 105,00g was applied to a 50 ml Affi-gel Blue (Bio-Rad) and the column was washed according to the published method. The purified NQO2 preparation was analysed by SDS-PAGE electrophoresis. The activity NQO2 in the presence of CB 1954, and various cofactors was determined by HPLC. To determine the kinetic parameters NQO2 (1μg/ml) was incubated with NRH (500μM) and CB 1954 at different concentrations (0.1 to 2 mM) in sodium phosphate buffer (10 mM, pH7) at 37°C. At various times, aliquots (10μl) were injected onto a Partisphere SCX (250x4.5mm) HPLC column (Whatman Ltd) and eluted isocratically (1.5ml/min) with 50 mM aqueous sodium phosphate containing 1% methanol. The eluate was continuously monitored for absorption at 320nm. This separation system could resolve all the

expected reduction products of CB1954 [Boland *et al*, 1991, Knox *et al*, 1992]. The reduction of CB 1954 was monitored by quantifying the increase in the area of the peak corresponding to the reduction product 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. All the assays were initiated by addition of the enzyme and performed in duplicate. The kinetic parameters were calculated by plotting the initial rate of reduction at each concentration of CB 1954 against that concentration and fitting the data to the Michales-Menton equation using a computer programme (FigP). Values were confirmed by transforming the data and fitting it to various linear forms of the equation by regression analysis.

The effect of various co-substrates on CB 1954 was determined as above but NADH. NADPH or NMNH was substituted for the NRH and CB 1954 was used at a fixed concentration of 100μM. The enzyme concentration was 5μg/ml. The reduction of CB 1954 was monitored by measuring both the decrease in its corresponding peak area on the HPLC trace and the increase in the area of the peak corresponding to the reduction product 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. The relative rates of reduction were determined at 10% reduction of CB1954 from a graph plotting CB 1954 reduction against time. The time axis was normalised to the equivalent of 10μg/ml of NQO2.

Table 1. Kinetic parameters for NQO2, *E. coli* nitroreductase, human and rat DT-diaphorase with respect to CB 1954. NRH was used as a co-substrate for NQO2 whilst the values for the other enzymes were determined using NADH.

ENZYME	Km (μM)	k_{cat} (s^{-1})
NQO2	263±13	6.01
Nitroreductase[1]	862±145	6.0
Rat DT-diaphorase[2]	826	0.0683
Human DT-diaphorase[2]	1403	0.0107

Data from: [1][Anlezark *et al*, 1992]

[2][Boland *et al*, 1991]

Table 2. The relative rate of CB 1954 reduction by NQO2 using different co-substrates. All co-substrates were used at an initial concentration of 500 μM and CB 1954 was at an initial concentration of 100 μM.

CO-SUBSTRATE	RELATIVE RATE OF REDUCTION
NADH	1.0
NADPH	1.24
NMNH	5.6
NRH	70.0

Example 2

In this example the prodrug is administered 6-48 hours following administration of a monoclonal antibody-NQO2 conjugate. The exact interval depends upon the localisation characteristics of the conjugate but prodrug administration ideally commences as soon as the plasma activity of enzyme is insufficient to catalyse enough prodrug to cause toxicity. The dose of conjugate is in the range 100-300 mg m^{-2} per patient. Administration of the cosubstrate NRH commences approximately 1 hour prior to the administration of prodrug and continues throughout the period of prodrug administration. The dose of prodrug depends upon its nature but an effective dose may be in the range 10-2000 mg m^{-2}. The dose of NRH may be 2-3 times the dose of prodrug. In this system it may be

advantageous to accelerate clearance of residual enzyme activity from plasma and normal tissues. This may be achieved by administration of a galactosylated anti-enzyme antibody following conjugate administration but prior to administration of NRH.

Example 3

In this example a recombinant polynucleotide is administered. Administration may be by any route appropriate to the condition to be treated, suitable routes including oral, nasal and parenteral. The dosage is determined by the individual clinicians for individual patients and this is determined by the exact nature of the prodrug and the cytotoxic agent to be released from the prodrug. Approximate doses are given in Example 2 above. When the expression of NQO2 is at a useful level administration of NRH followed by prodrug can commence as detailed in Example 2.

Example 4

In this example a patient is administered NRH and after 1 hour concurrent administration of prodrug is started. As previously, the doses of prodrug and NRH will depend upon the nature of the prodrug and the cytotoxic agent released from the prodrug.

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CLAIMS

1. A compound comprising a target cell-specific portion and human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug, or a polynucleotide encoding said NQO2 or said variant or fragment or fusion or derivative.

2. A compound according to Claim 1 comprising a target cell-specific portion and human NAD(P)H:quinone reductase 2 (NQO2).

3. A compound according to Claim 1 or 2 wherein the target cell-specific portion is tumour cell-specific.

4. A compound according to any one of Claims 1 to 3 wherein the target cell-specific portion comprises an antibody or fragment or derivative.

5. A compound according to any one of Claims 1 to 4 wherein the human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof is located substantially inside or following expression of the polynucleotide is located substantially inside the target cell.

6. A compound according to any one of Claims 1 to 5 comprising means for delivering said polynucleotide to said target cell.

7. A recombinant polynucleotide comprising a target cell-specific promoter operably linked to a polynucleotide encoding human

NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug.

8. A recombinant polynucleotide according to Claim 7 wherein said promoter is tumour cell-specific.

9. A recombinant polynucleotide according to Claim 7 or 8 comprising a polynucleotide encoding NQO2.

10. A recombinant polynucleotide according to any one of Claims 7 to 9 wherein following expression in the target cell the NQO2 or a variant or fragment or fusion or derivative thereof is located substantially inside the target cell.

11. A compound according to any one of Claims 1 to 6 wherein said polynucleotide is the recombinant polynucleotide of any one of Claims 7 to 10.

12. A therapeutic system comprising a compound according to any one of Claims 1 to 6 or 11, or a polynucleotide according to any one of Claims 7 to 10 and a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2.

13. A system according to Claim 12 wherein the prodrug is CB 1954 or an analogue thereof.

14. A system according to Claim 13 wherein the prodrug is CB 1954.

15. A system according to any one of Claims 12 to 14 further

comprising a cosubstrate for NQO2.

16. A system according to Claim 15 wherein the cosubstrate is nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

17. A method of treating a patient with a target cell to be destroyed the method comprising (a) administering to the patient a compound according to any one of Claims 1 to 6 or 11, or a recombinant polynucleotide according to any one of Claims 7 to 10; (b) allowing the NQO2 or a variant or fragment or fusion or derivative thereof to localize at, or be expressed in, the target cell; and (c) administering a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2.

18. A method according to Claim 17 wherein the patient has a tumour to be treated.

19. A method according to Claim 17 or 18 wherein the prodrug is CB 1954 or an analogue thereof.

20. A method according to Claim 19 wherein the prodrug is CB 1954.

21. A method according to any one of Claims 17 to 20 the method further comprising administering to the patient a cosubstrate for NQO2.

22. A method according to Claim 21 wherein the cosubstrate is nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

23. A compound according to any one of Claims 1 to 6 or 11, or a recombinant polynucleotide according to any one of Claims 7 to 10, for use in medicine.

24. Use of a compound according to any one of Claims 1 to 6 or 11, or a recombinant polynucleotide according to any one of Claims 7 to 10, in the manufacture of a medicament for treating a patient with a target cell to be destroyed.

25. Use as defined in Claim 24 wherein the patient has been, is being or will be administered a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2.

26. Use of a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 in the manufacture of a medicament for treating a patient with a target cell to be destroyed wherein the patient has been, is being or will be administered a compound according to any one of Claims 1 to 6 or 11, or a recombinant polynucleotide according to any one of Claims 7 to 10.

27. Use as defined in Claim 26 wherein the patient has a tumour to be treated.

28. A method of treating a human patient with a target cell to be destroyed wherein the target cell expresses NQO2 the method comprising administering to the patient a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 and nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

29. A method according to Claim 28 wherein the cytotoxic drug is CB 1954 or an analogue thereof.

30. A method according to Claim 28 or 29 wherein the analogue of NRH is able to permeate the target cell membrane.

31. A method according to any one of Claims 28 to 30 wherein the target cell is a tumour.

32. A method according to any one of Claims 28 to 31 the method further comprising determining, before administering the prodrug or NRH or an analogue thereof, whether the target cell to be treated expresses NQO2.

33. A therapeutic system comprising a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 and nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

34. Nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2 for use in medicine.

35. Use of nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2 in the manufacture of a medicament for treating a human patient with a target cell to be destroyed.

36. Use as defined in Claim 35 wherein the patient has been, is being or will be administered a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2.

37. Use of a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 in the manufacture of a medicament for treating a human patient with a target cell to be destroyed wherein the patient has been, is being or will be administered NRH or an analogue thereof which can pass reducing equivalents to NQO2.

38. Any novel method of treating cancer as herein disclosed.

ABSTRACT

THERAPEUTIC SYSTEMS

A compound comprising a target cell-specific portion and human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug, or a polynucleotide encoding said NQO2 or said variant or fragment or fusion or derivative.

A recombinant polynucleotide comprising a target cell-specific promoter operably linked to a polynucleotide encoding human NAD(P)H:quinone reductase 2 (NQO2) or a variant or fragment or fusion or derivative thereof which has substantially the same activity as NQO2 towards a given prodrug.

The compounds and polynucleotides are useful in a method of treating a patient in conjunction with a suitable prodrug.

A method of treating a human patient with a target cell to be destroyed wherein the target cell expresses NQO2 the method comprising administering to the patient a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 and nicotinamide riboside (reduced) (NRH) or an analogue thereof which can pass reducing equivalents to NQO2.

Figure 1

Figure 1. The bioactivation of CB 1954. The initial step is the reduction of CB 1954 by the enzyme DT diaphorase to form 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide. This hydroxylamine derivative can react with thioesters to produce DNA reactive species. It is postulated that this is the N-acetoxy derivative. The major product of this reaction is however 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide that does not react readily with DNA. Formation of 4-amino-5-(aziridin-1-yl)-2-nitrobenzamide is in competition with the production of DNA binding products.

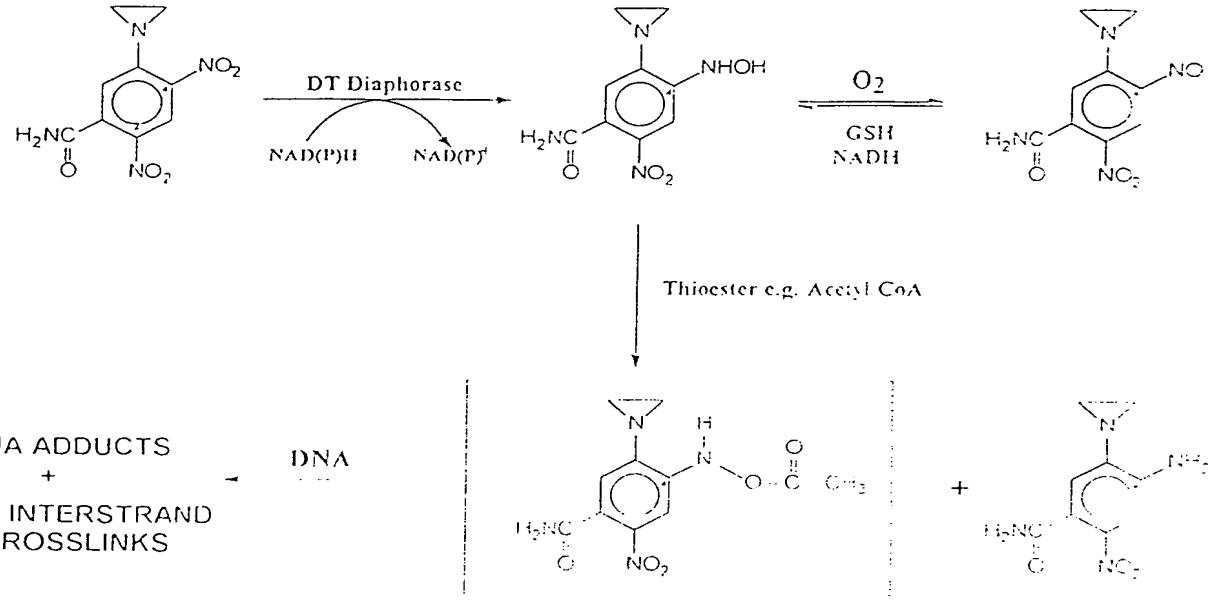
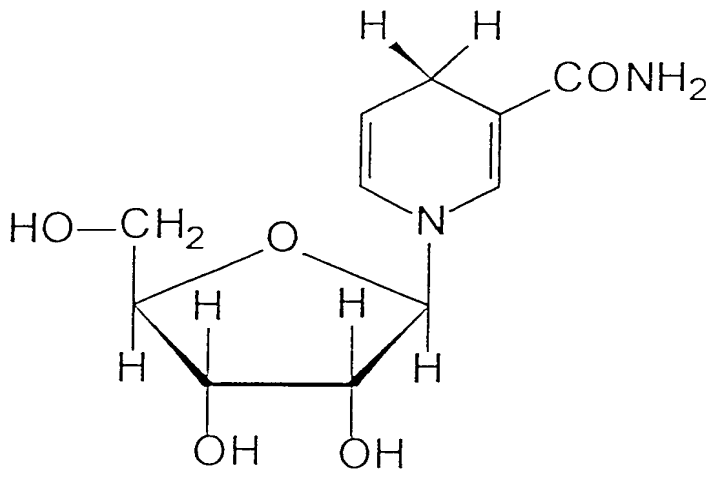


Figure 2. The structure of NRH.



$C_{11}H_{16}N_2O_5$
256.25
256.105921
C 51.6% H 6.3% N 10.9% O 31.2%

Figure 3. The bioactivation of CB 1954 by NQO2.

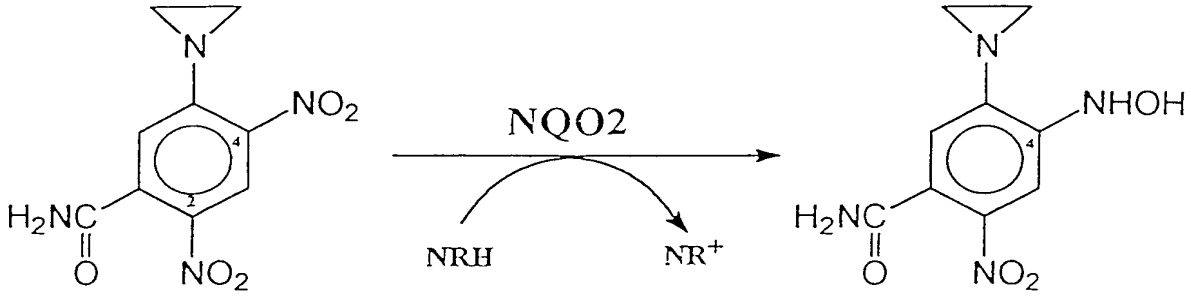


Figure 4. The reduction of CB 1954 by NQO2 in the presence of various co-substrates.

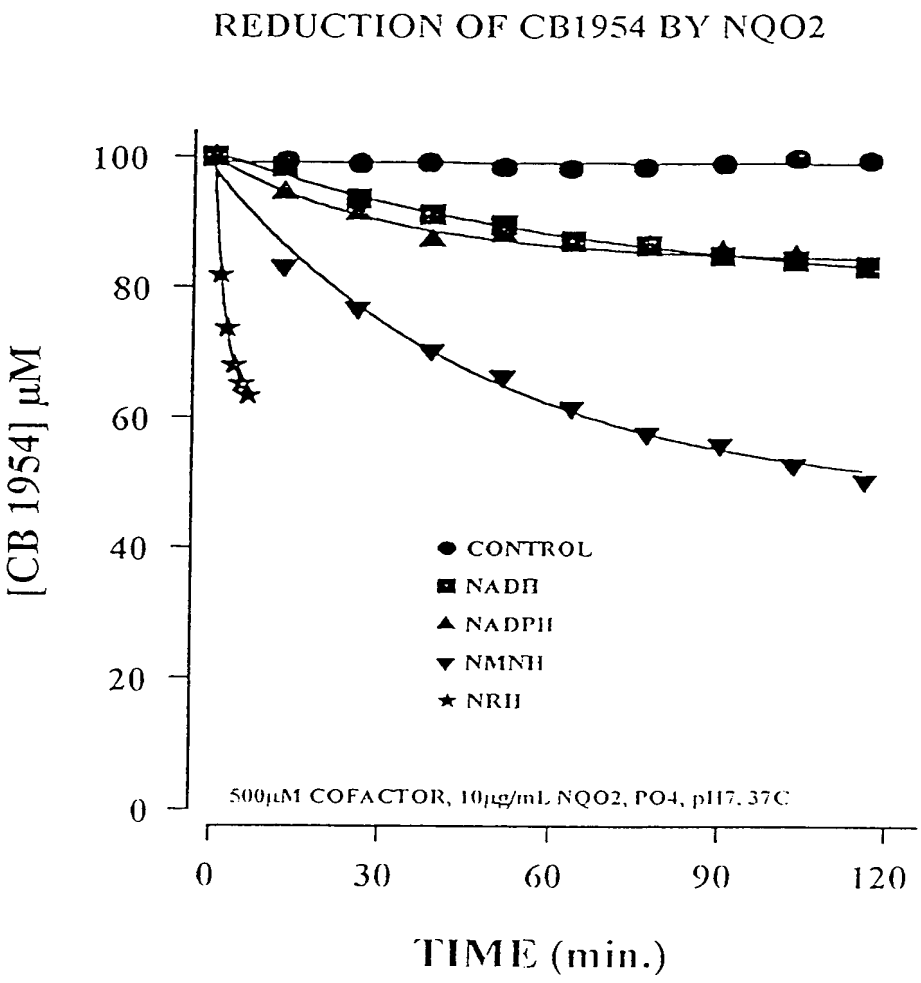
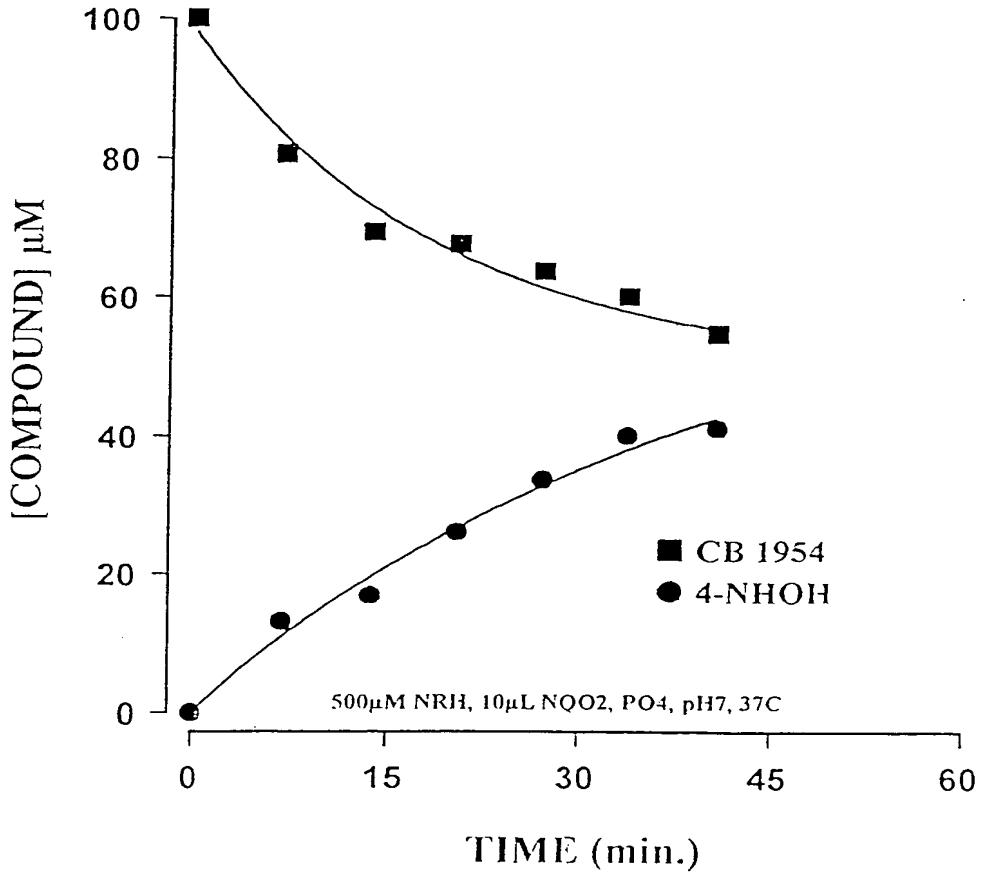


Figure 5. The formation of 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide (4-NHOH) from the reduction of CB 1954 by NQO2.



B Nucleic Acid Sequence of and Protein coded by HUMNQO2.SEQ

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M  A  G  K  K  V  L  I  V  Y  A  H  Q  E  P  K  S  F  N  G  S  L  K  N

    v         v         v         v         v         v         v
ATGTGGCTGTAGATGAACTGAGCAGGCAGGGCTGCACCGTCACAGTGTCTGATTTGTATGCCATGAACTT      315
  V  A  V  D  E  L  S  R  Q  G  C  T  V  T  V  S  D  L  Y  A  M  N  F        4

    v         v         v         v         v         v         v
TGAGCCGAGGGCCACAGACAAAGATATCACTGGTACTCTTTCTAATCCTGAGGTTTTCAATTATGGAGTG      385
 E  P  R  A  T  D  K  D  I  T  G  T  L  S  N  P  E  V  F  N  Y  G  V       70

    v         v         v         v         v         v         v
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E  T  H  E  A  Y  K  Q  R  S  L  A  S  D  I  T  D  E  Q  K  K  V  R  E

    v         v         v         v         v         v         v
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  A  D  L  V  I  F  Q  F  P  L  Y  W  F  S  V  P  A  I  L  K  G  W  M       11

    v         v         v         v         v         v         v
GGATAGGGTGCTGTGCCAGGGCTTTGCCTTTGACATCCCAGGATTCTACGATTCCGGTTTGCTCCAGGGT      595
 D  R  V  L  C  Q  G  F  A  F  D  I  P  G  F  Y  D  S  G  L  L  Q  G      140

    v         v         v         v         v         v         v
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K  L  A  L  L  S  V  T  T  G  G  T  A  E  M  Y  T  K  T  G  V  N  G  D      1

    v         v         v         v         v         v         v
ATTCTCGATACTTCCTGTGGCCACTCCAGCATGGCACATTACACTTCTGTGGATTTAAAGTCCTTGCCCC      735
  S  R  Y  F  L  W  P  L  Q  H  G  T  L  H  F  C  G  F  K  V  L  A  P       18

    v         v         v         v         v         v         v
TCAGATCAGCTTTGCTCCTGAAATTGCATCCGAAGAAGAAAGAAAGGGGATGGTGGCTGCGTGGTCCCAG      805
 Q  I  S  F  A  P  E  I  A  S  E  E  E  R  K  G  M  V  A  A  W  S  Q       210

    v         v         v         v         v         v         v
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R  L  Q  T  I  W  K  E  E  P  I  P  C  T  A  H  W  H  F  G  Q  <         231
```

Figure 6

PCT/GB98/01731